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**JC05 Rec'd PCT/PTO 15 SEP 2005****Method and kit for the specific detection of *M. tuberculosis***

The present invention relates to methods and means for the specific detection of *Mycobacterium tuberculosis* (*M. tuberculosis*) in a biological sample, the distinction between *M. tuberculosis* and the other members of the so-called *M. tuberculosis* complex, i.e. *Mycobacterium bovis* (*M. bovis*), *Mycobacterium bovis BCG* (*M. bovis BCG*), *Mycobacterium africanum* (*M. africanum*) and *Mycobacterium microti* (*M. microti*), in particular, being achieved.

At present, approximately 2 thousand million people worldwide have been infected with the pathogen of tuberculosis, *M. tuberculosis*, 8 million succumbing to tuberculosis every year. Of those suffering from tuberculosis, 3 million die every year. Consequently, tuberculosis is at present the bacterial infectious disease which most frequently leads to death. Because of the seriousness of the disease, tuberculosis is notifiable immediately after diagnosis in most industrialised nations not least in order to prevent spreading among the population as quickly as possible. In the last few years, an increased occurrence of tuberculosis has been observed both in the developing countries and in the industrialised nations, cases of death caused by it having been registered regularly above all in the case of immunosuppressed HIV patients. However, not only because of the general risk to large parts of the world population but also because of the meanwhile epidemic-type occurrence of multiply resistant bacteria strains (MDRTB) within or outside of hospitals, a rapid and clear-cut diagnosis for the detection of *M. tuberculosis* is required.

In man, tuberculosis is caused worldwide practically exclusively by the human tuberculosis pathogen *Mycobacterium tuberculosis*. The following need to be delimited from this: *Mycobacterium bovis* (*M. bovis*), the pathogen of cattle tuberculosis, *Mycobacterium bovis BCG*, an attenuated strain of *M. bovis* which is used for immunisation against tuberculosis and *Mycobacterium africanum* (*M. africanum*) and *Mycobacterium microti* (*M. microti*). As a result of their close relationship, all the above-mentioned species are combined in the so-called *M. tuberculosis* complex. From this, the so-called non-tuberculous mycobacteria need to be delimited. There are at present approximately 100 different species of these, at least 30 species of which occur in human material such as saliva, faeces, urine etc. and are capable in

some cases of leading also to infection and/or illnesses. The aim of the present efforts in the field of mycobacteria diagnosis is consequently not only the provision of mycobacteria tests which make it possible to recognise a mycobacteria infection within the shortest possible time at an early infection stage but also to specifically identify the members of the *M. tuberculosis* complex and to distinguish at the same time between *M. tuberculosis* and other members of the *M. tuberculosis* complex.

An identification of the members of the *M. tuberculosis* complex and the distinction between *M. tuberculosis* and the other members of the *M. tuberculosis* complex can normally be effected by testing the nitrate reductase activity and niacin accumulation of the mycobacteria (e.g. Metchock B.G. et al. In: Manual of Clinical Microbiology. ASM Press, Washington DC, 1999: 399-437). In this respect, *M. tuberculosis* is characterised, in comparison with the other members of the *M. tuberculosis* complex, by an increased nitrate reductase activity. In order to carry out the nitrate reductase test, however, the cultivation of the mycobacteria strains isolated from the clinical material, consequently in particular tuberculous pathogen strains, is above all necessary in the laboratory, which, normally, is possible only in laboratories specially equipped for the cultivation of highly infectious pathogens and, because of the slow growth of the pathogens, takes several weeks. The availability of such tests in clinical routine diagnosis is consequently associated with a high expenditure in terms of laboratory technology and finance.

All the detection methods existing so far are regarded as being unsatisfactory and consequently requiring improvement. No method suitable for clinical routine use which stands out in particular as a result of its simple application and by means of which it is possible to detect the members of the *M. tuberculosis* complex specifically in clinical material such as saliva, bronchial lavage, gastric juice, urine, faeces, bone marrow, blood or in biopsies and to distinguish simultaneously *M. tuberculosis* from the other members of the *M. tuberculosis* complex has so far become known from the state of the art.

It is known that the use of the real time PCR ("rapid cycle PCR") which is equipped with an air-tempered system and consequently exhibits considerably lower transition times compared with a conventional PCR, for example, leads to a substantially reduced time up to the detection of e.g. mycobacteria by amplification of the isolated genetic material of the mycobacterium (Chapin and Lauderdale, J. Clin. Microbiol. (1997) 35:2157-2159). In

addition, fluorimetric measurements represent a rapid and sensitive method for the detection of amplified gene fragments when using colour-labelled hybridisation probes, in particular within the framework of rapid cycle PCR. So far however, it has not been possible to provide a (real time) PCR test by means of which the members of the *M. tuberculosis* complex are specifically detected and, simultaneously, *M. tuberculosis* is distinguished from the other members of the *M. tuberculosis* complex.

Against this background, it is the object of the present invention to provide in particular improved methods and means which permit essentially a particularly rapid and simultaneously specific detection of members of the *M. tuberculosis* complex and by means of which it is simultaneously possible to distinguish *M. tuberculosis* from the other members of the *M. tuberculosis* complex.

According to the invention, the object is achieved by the methods according to claims 1 to 11, primer and primer pairs according to claims 12-17, hybridisation probes and hybridisation probe pairs according to claims 19 to 28, by the use according to claims 18 and 29 and in particular by kits according to claims 30 to 35 and/or the subject matter of claims 36 of 51.

The present invention relates in particular to a method for the specific detection of *M. tuberculosis* in a biological sample in which method a nucleic acid amplification method is carried out using primers which are suitable for amplifying a DNA segment from the sequence shown in SEQ ID NO: 1 which sequence comprises a segment from the region of the *narGHJI* nitrate reductase operon, the DNA segment comprising position -215 in the 5' to 3' direction of reading upstream of the translation start codon GTG of the *narGHJI* nitrate reductase operon

and in the case of which the polymorphism specific for *M. tuberculosis* is detected in position -215 in the 5' to 3' direction of reading upstream of the translation start codon GTG of the *narGHJI* nitrate reductase operon.

*NarGHJI* nitrate reductase operon should be understood to mean, according to the invention, a nucleic acid sequence, including the regulatory sequences, in particular including its promoter, which encodes *narGHJI* nitrate reductase. The polymorphism specific for *M. tuberculosis* is an individual nucleotide exchange (T->C), i.e. an SNP (single nucleotide

polymorphism) specific in comparison with the nucleic acid sequences of the other members of the *M. tuberculosis* complex, i.e. *M. bovis* BCG, *M. africanum* and *M. microti*.

According to an embodiment of the invention, microbial DNA is extracted, in a first step, from the sample which preferably contains clinical material and, in a further step, at least one DNA fragment of the nitrate reductase operon of mycobacteria, i.e. the *narGHJI* operon which encodes nitrate reductase is amplified from the extracted DNA, the amplified DNA fragment – or one of the amplified DNA fragments (insofar as representatives of several mycobacteria strains are present in the biological sample) – containing a target region (target sequence) which exhibits at least one DNA polymorphism specific for *M. tuberculosis*.

In a further step, the specific hybridisation of the at least one amplified DNA fragment is detected with at least one hybridisation probe, i.e. oligonucleotide, the hybridisation probe comprising a nucleotide sequence which is preferably selected from the group consisting of the nucleotide sequence represented in SEQ ID No: 5, the complementary sequence to SEQ ID NO: 5, the nucleotide sequence represented in SEQ ID NO: 6 and the complementary sequence to SEQ ID NO: 6, the specific detection of *M. tuberculosis* vis-à-vis other members of the *M. tuberculosis* complex, i.e. above *M. bovis*, BCG, *M. africanum* and *M. microti* taking place by analysis of the melting temperature of the specific hybridisation of the hybridisation probe with the amplified DNA fragment of the *narGHJI* operon, i.e. by melting curve analysis. According to a particular embodiment, the probe/probes exhibits/exhibit the above-mentioned nucleotide sequence/nucleotide sequences. According to a particular embodiment, the above-mentioned hybridisation probes are combined with the anchor probe according to SEQ ID NO: 4 or the nucleotide sequence complementary therewith.

According to the invention, the at least one *M. tuberculosis*-specific DNA polymorphism of the *narGHJI* operon is preferably located in position -215 in the 5' to 3' direction of reading upstream of the translation start codon GTG of the *narGHJI* operon, i.e. in the promoter region, hereinafter called *narGHJI* promoter. Types of *M. tuberculosis* formed by mutation which exhibit this polymorphism (T in comparison with C in the case of the other members of the *M. tuberculosis* complex) in the *narGHJI* promoter are included even if this is not positioned in position -215 but at a position which differs with respect to the translation start codon GTG of the *narGHJI* operon.

Surprisingly enough, it has been found within the framework of the present invention that, instead of the 215<sup>th</sup> nucleotide in the 5' to 3' direction of reading upstream of the translation start codon GTG of the *narGHJI* operon, i.e. in position -215 of the *narGHJI* promoter, a thymine base is to be found in the case of *M. tuberculosis* whereas a cytosine base is present in the case of the other members of the *M. tuberculosis* complex (in particular *M. africanum*, *M. microti*, *M. bovis* and *M. bovis* (BCG) (compare Fig. 1). This single base polymorphism is specific for *M. tuberculosis* and is, above all, stable as confirmed by a comparative investigation into more than 100 *M. tuberculosis* strains worldwide. Moreover, it has been found unexpectedly that this one nucleotide polymorphism is responsible for the different reactions in the nitrate reductase test of *M. tuberculosis* in comparison with the other members of the *M. tuberculosis* complex (compare example 4).

According to the invention, at least one primer, in particular at least one primer pair is preferably used for the amplification of the DNA fragment of the *narGHJI* operon, the primers containing, i.e. comprising, in particular, however, consisting of, i.e. exhibiting, the nucleotide sequence represented in SEQ ID NO: 2 and/or the nucleotide sequence represented in SEQ ID NO: 3. Preferably, according to the invention, the at least one pair of labelled hybridisation probes preferably used for the specific hybridisation of the amplified DNA fragment contains, in particular it consists of, nucleotide sequences selected from the group of the nucleotide sequence pairs of the nucleotide sequences represented in SEQ ID NO: 4 and in SEQ ID NO: 5, the pair of complementary sequences of SEQ ID NO: 4 and SEQ ID NO: 5, the nucleotide sequences represented in SEQ ID NO: 4 and in SEQ ID NO: 6 and the pair of complementary sequences of SEQ ID NO: 4 and SEQ ID NO: 6.

A clear-cut detection of a tuberculous infection by pathogen strains of the type *M. tuberculosis*, i.e. in particular the strains of *M. tuberculosis* H37v (ATCC 25618) and *M. tuberculosis* Erdmann (ATCC 35801) and other clinical strains takes place, according to the invention, preferably by the analysis of the melting temperatures of the specific hybridisation of a labelled hybridisation probe pair with the nucleotide sequences SEQ ID NO: 4 and SEQ ID NO: 5 or the pair of complementary sequences with a *narGHJI* promoter region, specifically for *M. tuberculosis*. In this respect, *M. tuberculosis* stands out by melting temperatures of in particular less than 61°C, preferably less than 59°C, particularly preferably of approximately 57°C. In comparison, the pathogen strains of the mycobacteria types *M. africanum*, *M. microti*, *M. bovis* and *M. bovis* BCG to be distinguished from *M. tuberculosis*,

possess, when the labelled hybridisation probe pair SEQ ID NO: 4/ SEQ ID NO: 5 is used, melting temperatures of, in particular, more than 59°C, preferably of more than 61°C, particularly preferably of more than 63°C.

Depending on the hybridisation conditions actually prevailing, e.g. on the buffer composition, the design of the probes as labelled probes or by modification of the nucleotide sequence of the probes, (usually slight) changes to the absolute melting temperature may arise; obviously, all those methods in the case of which such modified melting temperatures occur represent preferred embodiments of methods according to the invention presented here.

In a further variant, the clear-cut detection of a tuberculous infection by pathogen strains of the type of *M. tuberculosis*, i.e. in particular the strains of *M. tuberculosis* H37v (ATCC 25618) and *M. tuberculosis* Erdmann (ATCC 35801) as well as further clinical strains takes place according to the invention preferably by the analysis of the melting temperatures of the specific hybridisation of a labelled hybridisation probe pair with the nucleotide sequences SEQ ID NO: 4 and SEQ ID NO: 6 or the pair of the complementary sequences with a *narGHJI* promoter region specific for *M. tuberculosis* (compare Table 1). The pathogen strains of the type of *M. tuberculosis* are characterised in this respect by melting temperatures of in particular more than 58°C, preferably more than 60°C, particularly preferably of approximately 62°C. In comparison, the pathogen strains of *M. africanum*, *M. microti*, *M. bovis* and *M. bovis* BCG, when the labelled hybridisation probe pair SEQ ID NO: 4/ SEQ ID NO: 6 is used, possess melting temperatures of in particular less than 62°C, preferably less than 60°C, particularly preferably of approximately 58°C.

The nucleic acid amplification according to the invention can take place, for example, by Polymerase Chain Reaction (PCR), Nucleic Acid Sequence Based Amplification (NASBA), Strand Displacement Amplification (SDA) or Ligase Chain Reaction (LCR). In addition, any desired further nucleic acid amplification methods can obviously be considered as suitable.

Moreover, it has, surprisingly, been found that when using at least one primer, in particular one primer pair comprising the nucleotide sequence represented in SEQ ID NO: 2 and/or comprising the nucleotide sequence represented in SEQ ID NO: 3 for the amplification of at least one DNA fragment of the promoter region of the *narGHJI* nitrate reductase operon of mycobacteria, the amplification takes place exclusively in the case of the pathogen strains of

the *M. tuberculosis* complex, i.e. leading to an amplification product. In the case of non-tuberculous pathogens or non-mycobacterial pathogens, however, no amplification takes place which means that no corresponding amplification product is obtained. By using the primers according to the invention, the clear-cut distinction between the pathogen strains *M. tuberculosis* and the other pathogen strains of the *M. tuberculosis* complex (compare above) as well as vis-à-vis non-tuberculous mycobacteria and non-mycobacteria, for example actinomycetes, pathogens of the genus *Bacillus*, *Staphylococcus*, *Listeria*, *Enterococcus* and *Proteus*, *E. coli*, *Salmonella*, *Shigella*, *Klebsiella* or *Pseudomonas* or fungal pathogens is possible particularly advantageously.

In a preferred embodiment of the above-mentioned methods, the amplification of the DNA fragments by Polymerase Chain Reaction (PCR) is carried out. In a particularly preferred embodiment, the PCR is a real time PCR (rapid cycle PCR).

The detection of the polymorphism specific for *M. tuberculosis* can take place, according to the invention, by the specific hybridisation of one or several probes.

In the real time PCR method, it is possible to observe the multiplication of the PCR products in real time, amplification cycle by amplification cycle. Particularly preferably, the amplification is carried out in LightCycler™ system from Roche Molecular Biochemicals which is an embodiment of real time PCR. For this purpose, hybridisation probes which bind specifically to the desired PCR amplification products, in particular, are also added to the PCR starting mixture, apart from polymerase, the nucleotides, the buffer solutions and the primers. In this connection, two sequence-specific oligonucleotide probes are used in particular which are labelled with different dyes. The sequences of the hybridisation probe pairs labelled according to the invention are selected in such a way that they hybridise to the target sequence of the amplified DNA fragment in such a way that the 3' end of the one probe is situated close to 5' end of the other probe as a result of which the two dyes are brought into direct vicinity to each other; preferably, the distance between the two probes is between 1 and 5 nucleotides. In particular, a fluorescence resonance energy transfer (FRET; compare e.g. Heid et al, Genome Res. 6 (1996) 986-994) between the two dyes of the hybridisation probes and consequently a shift in the fluorescence spectrum occurs, the degree of fluorescence in this wave length region being a function of the quantity of detected DNA. The detection can take place according to the method according to WO 00/58505, for example.

According to the invention, the FRET system also provides for quantitative measurements of the quantity of amplified DNA fragments. The hybridisation probes selected according to the invention can bind quantitatively, i.e. stoichiometrically, to the amplified fragments. In this respect, the quantitative hybridisation is dependent in particular on the temperature and the degree of homology of the oligonucleotide probes used with the detected sequence on the amplified DNA fragment.

In a preferred embodiment, the fluorimetric detection of specific DNA sequences in the amplified fragments is carried out after amplification of the fragments by conventional PCR. In an embodiment which is particularly preferred, the fluorimetric detection is carried out in a rapid cycle PCR during the amplification reactions in which, for example, the increase in DNA produced can be monitored as an increase in the fluorescence signal.

In a preferred embodiment of the method according to the invention, the specific detection of the amplified DNA fragments takes place on completion of the amplification reaction, wherein, within the framework of a melting curve analysis, the temperature being modified, preferably increased continuously, following the hybridisation of the hybridisation probe pair, preferably of a FRET pair, to the region to be detected, and the fluorescence emitted simultaneously as a function of the temperature being measured. In this way, a melting temperature is determined at which the hybridisation probes, in particular the FRET pair used, just no longer hybridise to the region, to be detected, of the amplified DNA fragment. The essential aspect of a melting curve analysis consists of the fact that, in the case of the occurrence of mismatches between the hybridisation probe pair used and the target region on the amplified DNA fragment, a reduction of the melting point measured takes place. Using hybridisation probes, in particular a FRET pair, the regions of DNA fragments are identified according to the invention in this way whose sequences differ only very slightly from each other by one or several point mutations in the nucleotide sequence.

In connection with the present invention, the terms "segment", "region", "fragment", "target region" or "flanking region" should be understood to mean at least one coherent area on a linear, strand-type deoxy or ribonucleic acid molecule, i.e. DNA or RNA, which, measured in the number of nucleotides of this molecule, consists, in the 5' to 3' direction of reading

downstream and/or upstream of a certain numbered nucleotide of the molecule, i.e. a certain position, preferably of 200, 100, 50, 40, 30, 20, 10, 5, 4, 3 or 2 nucleotides of the molecule.

A DNA segment amplified according to the invention preferably has, in addition to the sequence to which the primer binds, a length of at least 1 and maximum 500 nucleotides, preferably maximum 300 and particularly preferably maximum 155 nucleotides. Individual length values which are within the above-mentioned region, are expressly included, i.e. 2, 3, 4, 5 ...., 497, 498 and 499. The amplified DNA segment can obviously be longer, shorter segments in practice being probably preferred.

According to an embodiment of the invention, the primer pair used for the method has at least one of the sequences with SEQ ID NO: 2 and SEQ ID NO: 3 or the complementary sequences thereof.

In an embodiment of the invention, the detection of the polymorphism specific for *M. tuberculosis* takes place with at least one pair of labelled hybridisation probes, one probe being labelled at its 3' end and the other probe at its 5' end and the probes binding specifically to the amplificate in such a way that a fluorescence resonance energy transfer (FRET) is made possible (compare above).

In a preferred embodiment, the probe pair exhibits the sequences with the SEQ ID NO: 4 and 5 or the complementary sequences thereof and/or the sequences with the SEQ ID NO: 4 and 6 or the complementary sequences thereof.

The subject matter of the invention is, moreover, a method for the joint detection of pathogens of the *M. tuberculosis* complex in clinical materials, the presence of an amplification product of the *narGHJI* promoter region being detected in particular by at least one hybridisation probe specific for the amplified DNA fragment, preferably by the above-described method and pathogen strains of the *M. tuberculosis* complex thus being detected vis-à-vis non-tuberculous pathogens and/or vis-à-vis non-mycobacteria. In an alternative variant, the occurrence of an amplification product is detected in a manner known as such, in particular by electrophoretic methods. In this connection, the method of the present invention can be part of a comprehensive detection method in which further, preferably pathogen-specific (type specific or species specific) detections are carried out by nucleic acid

amplification corresponding to specific sequence segments in parallel batches or in the same reaction batch, in particular in connection with a multiplex PCR.

An essential advantage of the method according to the invention consists of the fact that, in a detection method for the diagnosis of mycobacteria infections, both an existing infection with *M. tuberculosis* can be clearly, accurately and in particular simultaneously recognised, preferably in a single routine diagnostic batch vis-à-vis other microbial infections, an infection with *M. tuberculosis* vis-à-vis non-tuberculous infections and in infection with *M. tuberculosis* vis-à-vis other members of the *M. tuberculosis* complex. A further essential advantage of the method according to the invention is that, the mycobacteria strain of the type of *M. tuberculosis* can be detected additionally, in particular simultaneously and in a clear-cut manner and this type can be identified individually. The detection methods according to the invention consequently allow in particular a clear-cut and accurate early recognition of tuberculosis and the clear-cut and accurate early recognition of the type-dependence to *M. tuberculosis* of isolated pathogen strains of the *M. tuberculosis* complex. This permits particularly advantageously a rapid and controlled therapy of the infected organism.

Essentially, the method according to the invention is used in routine diagnosis and essentially in routine laboratories. Carrying out the method in specially equipped safety laboratories of complex design to cultivate highly infectious pathogen strains is not necessary. For this reason, the method according to the invention can be made available to a wide range of users.

In connection with the present invention, clinical materials should be understood to mean essentially clinical samples, i.e. patient material such as saliva, bronchial lavage, gastric juice, urine, faeces, liquor, bone marrow, blood but also biopsies, in particular aspirate biopsies such as e.g. from the lymph nodes in the neck. However, clinical material should also be understood to mean culture isolates e.g. from liquid culture, in particular from liquid culture for the selective cultivation of acid resistant rod cells, in particular from patient material.

Preferably, the microbial DNA is extracted from the clinical material in a manner known as such, in particular by means of DNA preparation kits such as the QIAamp<sup>TM</sup> DNA Mini Kit from Qiagen.

Preferably, the sample according to the invention is selected from the group of clinical samples selected consisting of saliva, bronchial lavage, gastric juice, urine faeces, liquor, bone marrow, blood and biopsies.

The present invention also relates to the primers, primer pairs, probes and probe pairs required for the execution of the above-mentioned methods.

A primer pair which is suitable for the amplification of a DNA segment from the sequence shown in SEQ ID NO: 1 which comprises a segment from the area of the *narGHJI* nitrate reductase operon is preferred according to the invention, the DNA segment comprising position -215 in the 5' to 3' direction of reading upstream of the translation start codon GTG of the *narGHJI* nitrate reductase operon.

According to the invention, the primer pair is preferably used for the amplification of a DNA fragment with a DNA polymorphism of the *narGHJI* gene specific for pathogen strains of the type *M. tuberculosis* from the extracted DNA with a length of 155 bp, in the case of which the forward primer comprises and/or exhibits the nucleotide sequences according to SEQ ID NO: 2 and the reverse primer the nucleotide sequence according to SEQ ID NO: 3. A further subject matter of the invention consequently also consists of at least one oligonucleotide primer, preferably at least one oligonucleotide primer pair, for the amplification of a DNA fragment of the *M. tuberculosis*-specific promoter region of the *narGHJI* nitrate reductase gene containing, in particular consisting of, nucleic acid molecules with nucleotide sequences represented in SEQ ID NO: 2 and/or SEQ ID NO: 2 or comprising these nucleotide sequences.

In a preferred embodiment, at least one primer of a primer pair exhibits the sequence indicated in SEQ ID NO: 2 or the complementary sequence thereof or comprises it.

In a further preferred embodiment, at least one primer of a primer pair exhibits the sequence indicated in SEQ ID NO: 3 or the complementary sequence thereof or comprises it.

In a particularly preferred embodiment, the primers of a primer pair exhibit the sequences indicated in SEQ ID NO: 2 and those indicated in SEQ ID NO: 3 or the complementary sequences thereof.

Primers according to the invention may exhibit the sequence indicated in SEQ ID NO: 2 or the complementary sequence thereof or the sequence indicated in SEQ ID NO: 3 or the complementary sequence thereof.

An embodiment of the invention relates to the use of one the above-mentioned primers or primer pairs for the specific detection of *M. tuberculosis*, i.e. by amplification of *M. tuberculosis* DNA contained in a biological sample and the specific detection of the amplicate obtained (compare above).

The invention preferably relates also to primers and/or primer pairs for the amplification of the DNA fragments used according to the invention which, compared with the above-mentioned primers according to the invention comprising the nucleotide sequences represented in SEQ ID NO: 2 and 3, degenerated, mutated or modified sequences or fragments thereof in each case, which hybridise with the nucleotide sequence concerned represented in SEQ ID NO: 2 and 3 from which they are derived, a degree of homology existing in each case preferably over the entire length of the sequence of at least 92%, preferably of at least 97%, particularly preferably of at least 98% compared with the original nucleotide sequence. In this respect, the derived fragments have a sequence length in each case which is preferably maximum 98% of the length the nucleotide sequence, particularly preferably maximum approximately 95%, maximum approximately 90%, maximum approximately 75%, maximum approximately 50% or maximum approximately 25%. Particularly preferably, the derived fragment is maximum 10, maximum 5, 4, 3 or 2 nucleotides or one nucleotide shorter compared with the original nucleotide sequence.

In connection with the present invention, the term "modified sequence" or "modified nucleotide sequence" should be understood to be a nucleic acid sequence which, by exchange, inversion, deletion or addition of at least one nucleotide, including an unusual or synthetic nucleotide, differs from its original sequence in at least one nucleotide, preferably in two nucleotides. In this connection, the term "modified" should be understood to mean a characteristic which relates to a modified nucleotide sequence.

In connection with the present invention, the wordings "primer which comprises the nucleotide sequence" or "hybridisation probe which comprises the nucleotide sequence" or

similar should be understood to mean that the primers and probes concerned exhibit the nucleotide sequences, i.e. consist of the specifically mentioned nucleotide sequences alone. The wording should also be understood to mean that the primers and probes concerned consist, if necessary, of at least one further additional sequence, apart from the nucleoside sequences mentioned in concrete terms. This additional sequence flanks the nucleotide sequences mentioned in concrete terms and has a sequence length which amounts to preferably maximum approximately 100% of the length of the nucleotide sequence mentioned in concrete terms, particularly preferably maximum approximately 75%, maximum approximately 50%, maximum approximately 25%, maximum approximately 10%, maximum approximately 5% or maximum approximately 2%. Particularly preferably, the additional sequence has a length of 10 to 5 nucleotides, of 4, 3, 2 nucleotides or it consists of a single nucleotide.

A hybridisation probe according to the invention is a probe which is suitable for the specific detection of the polymorphism specific for *M. tuberculosis* which is located in position -215 in the 5' to 3' direction of reading upstream of the translation start codon GTG of the *narGHJI* nitrate reductase operon.

The hybridisation probe pair according to the invention is a probe pair which is suitable for the specific detection of the polymorphism specific for *M. tuberculosis* which is located in position -215 in the 5' to 3' direction of reading upstream of the translation start codon GTG of the *narGHJI* nitrate reductase operon.

A further subject matter of the invention also consists of at least one oligonucleotide hybridisation probe which hybridises specifically with an *M. tuberculosis*-specific promoter region of the *narGHJI* nitrate reductase operon which contains, in particular consists of, the nucleic acid molecule with the nucleotide sequence represented in SEQ ID NO: 5 or with the complementary sequence thereof. A further subject matter of the invention also consists of at least one oligonucleotide hybridisation probe pair which contains, in particular consists of, the nucleic acid molecules with the nucleotide sequences represented in SEQ ID NO: 4 and in SEQ ID NO: 5 or with the complementary sequences thereof.

A further subject matter of the invention also consists of a further or alternative oligonucleotide hybridisation probe which hybridises specifically with an *M. tuberculosis*-

specific promoter region of the *narGHJI* nitrate reductase operon which contains, in particular consists of, the nucleic acid molecule with the nucleotide sequence represented in SEQ ID NO: 6 or with the complementary sequence thereof. A further subject matter of the invention also consists of at least one oligonucleotide hybridisation probe pair which contains, in particular consists of, the nucleic acid molecules with the nucleotide sequences represented in SEQ ID NO: 4 and in SEQ ID NO: 6 or with the complementary sequences thereof.

In a particularly preferred embodiment, at least one probe of a hybridisation probe pair exhibits the sequence indicated in SEQ ID NO: 4 or the complementary sequence thereof or the sequence indicated in SEQ ID NO: 5 or the complementary sequence thereof or the sequence indicated in SEQ ID NO: 6 or the complementary sequence thereof.

In a particularly preferred embodiment, the probes of a hybridisation probe pair exhibit the sequences indicated in sequence SEQ ID NO: 4 and SEQ ID NO: 5 or the complementary sequences thereof or the sequences indicated in sequence SEQ ID NO: 4 and SEQ ID NO: 6 or the complementary sequences thereof.

Hybridisation probes according to the invention may exhibit the sequence indicated in SEQ ID NO: 4 or the complementary sequence thereof or the sequence indicated in SEQ ID NO: 5 or the complementary sequence thereof or the sequence indicated in SEQ ID NO: 6 or the complementary sequence thereof.

In a manner preferred according to the invention, the detection of the DNA fragments is carried out with the above-mentioned hybridisation probes which are executed as FRET pairs, i.e. labelled, a hybridisation probe partner being executed as donor component = "anchor probe" which is associated preferably at the 3' terminal nucleotide with a dye, preferably a fluorescence dye, particularly preferably with fluorescein. According to the invention, the hybridisation probes comprising the nucleotide sequence represented in SEQ ID NO: 4 or the complementary sequence thereof are provided preferably as anchor probes.

The hybridisation probe partner, which is different in each case, of the hybridisation probe pair is executed as acceptor component = sensor probe which is preferably associated at the 5' terminal nucleotide with a further dye, preferably with a rhodamine derivative. According

to the invention, the hybridisation probes comprising the nucleotide sequences represented in SEQ ID NO: 5, 6 or the complementary sequences thereof in each case are preferably provided as sensor probes. In preferred variants of the above-mentioned embodiments, the rhodamine derivative is LightCycler-Red 640®, in further preferred variants of the above-mentioned embodiment the rhodamine derivative is LightCycler-Red 705®, in further preferred variants of the above-mentioned embodiment the rhodamine derivative is Cy5. Particularly preferably, the labelled sensor probes used for the type-specific detection of *M. tuberculosis* according to the invention carry the dye LightCycler-Red 640 ® or LightCycler-Red 705®.

A further embodiment of the invention relates to the use of one of the hybridisation probes described above or one of the hybridisation probe pairs described above for the specific detection of *M. tuberculosis*.

Obviously, the present invention also comprises means such as e.g. kits (devices) for carrying out a method according to the invention.

In one embodiment, a kit comprises at least one primer pair which is suitable for the amplification of a DNA segment from the sequence shown in SEQ ID NO: 1 which comprises a segment from the area of the *narGHJI* nitrate reductase operon, the DNA segment comprising position -215 in the 5' to 3' direction of reading upstream of the translation start codon GTG of the *narGHJI* nitrate reductase operon and/or at least one hybridisation probe or one hybridisation probe pair which is suitable for the specific detection of the polymorphism specific for *M. tuberculosis* which is located at position -215 in the 5' to 3' direction of reading upstream of the translation start codon GTG of the *narGHJI* nitrate reductase operon.

In a particularly preferred embodiment, the kit comprises at least one primer pair in the case of which the primers exhibit the sequences indicated in SEQ ID NO: 2 and SEQ ID NO: 3 or the complementary sequences thereof.

In a further preferred embodiment, the kit comprises at least one hybridisation probe pair in which the probes exhibit SEQ ID NO: 4 and SEQ ID NO: 5 or the complementary sequences thereof or SEQ ID NO: 4 and SEQ ID NO: 6 or the complementary sequences thereof.

In a particularly preferred embodiment, the kit comprises both a primer pair in the case of which the primers exhibit the sequences indicated in SEQ ID NO: 2 and SEQ ID NO: 3 or the complementary sequences thereof as well as a hybridisation probe pair in which the probes exhibit the SEQ ID NO: 4 and SEQ ID NO: 5 or the complementary sequences thereof or the SEQ ID NO: 4 and SEQ ID NO: 6 or the complementary sequences thereof.

In a further embodiment, the kit additionally comprises further equipment, reagents and/or auxiliary agents necessary for carrying out a nucleic acid amplification and/or detection reaction of the amplicate.

A further subject matter of the invention also consists of the use of at least one *M. tuberculosis*-specific DNA polymorphism in the promoter region of the *narGHJI* nitrate reductase operon of mycobacteria, in particular in position -215 in the 5' to 3' direction of reading upstream of the translation start codon GTG of the *narGHJI* operon for the specific detection of an infection with *M. tuberculosis*. In a particularly preferred variant, this genome segment is characterised in that it contains, in particular consists of, the nucleic acid sequence represented in SEQ ID NO: 1 or the complementary sequence. Preferably, the use according to the invention takes place in at least one PCR batch with subsequent or simultaneous hybridisation, e.g. in a rapid cycle PCR or in a method of conventional hybridisation known as such of DNA sequencing, in particular in a capillary sequencing device; namely allele-specific PCR, OLA ("Oligonucleotide Ligation Assay"), SSCP ("Single Strand Conformation Polymorphism") or Denatured Gradient Gel Electrophoresis (DGGE). Alternative methods for amplification consist, apart from PCR, for example of the known methods of NASBA, SDA or LCR.

The invention will be explained in further detail by way of the appended sequence protocol which contains the sequences No. 1 to 6, by way of Figures 1 to 5 and by way of examples 1 to 4.

SEQ ID NO: 1 – region in the area of the *narGHJI* operon which comprises the polymorphism specific for *M. tuberculosis* in position -215 in the *narGHJI* promoter.

SEQ ID NO: 2 – forward primer for the amplification of a 155 bp fragment of the *narGHJI* promoter of mycobacteria containing a DNA polymorphism specific for *M. tuberculosis*,

SEQ ID NO: 3 – reverse primer to SEQ ID NO: 2;

SEQ ID NO: 4 – hybridisation probe (antisense), in particular anchor probe and donor component of a probe pair for the detection of the type-specific region of the *narGHJI* promoter of mycobacteria;

SEQ ID NO: 5 – hybridisation probe (antisense), in particular sensor probe and acceptor component of a probe pair for the detection of the type-specific region of the *narGHJI* promoter of mycobacteria;

SEQ ID NO: 6 – hybridisation probe (antisense), in particular sensor probe and acceptor component of a probe pair for the detection of the type-specific region of the *narGHJI* promoter of mycobacteria;

#### Description of the figures

Figure 1 – shows an alignment of the *narGHJI* promoter regions of *M. tuberculosis*, *M. bovis* and *M. bovis BCG*. The labelled area shows the nucleotide polymorphism specific for *M. tuberculosis* (“T”).

Figure 2 – shows melting curves of the hybridisation of the specific hybridisation probe pair with SEQ ID NO: 4/SEQ ID NO: 5 with the region, specific for *M. tuberculosis*, in the amplified 155 bp fragment of the *narGHJI* promoter.

Figure 3 – shows melting curves of the hybridisation of the specific hybridisation probe pair with SEQ ID NO: 4/SEQ ID NO: 6 with the region, specific for *M. tuberculosis*, in the amplified 155 bp fragment of the *narGHJI* promoter.

Figure 4 – shows a diagnostic nitrate reductase test with specifically produced T-215C mutants of *M. tuberculosis*, the corresponding Wild types and *M. bovis* and *M. bovis BCG*. The red colouration (dark) by the diazonium dye formed from naphtyl amide and sulphanyl

acid indicates the nitrate accumulation in the medium: the Wild types of *M. tuberculosis* alone exhibit a nitrate accumulation; the T-215C mutants thereof posses (again) the *M. bovis* phenotype.

Figure 5 – shows an area of the *narG* gene (SEQ ID NO: 1) which exhibits the nucleotide polymorphism specific for *M. tuberculosis* (T) in position -215 upstream of the translation start codon GTG.

### Examples

#### Example 1: DNA isolation

##### a) from clinical material

Microbial DNA is purified, i.e. extracted, from clinical samples consisting of saliva, bronchial lavage, gastric juice, urine, faeces, liquor, bone marrow, blood or aspirate biopsies in the known way, e.g. by means of a QIAamp<sup>TM</sup> DNA Mini Kit (Qiagen, Hilden, Germany).

##### b) from culture isolates

Cultures from microorganisms, to be diagnosed, from patient samples are cultivated in an automated cell culture system BACTEC<sup>TM</sup> MGIT<sup>TM</sup> (Becton, Dickinson and Company Diagnostic Systems, USA) in liquid cultures under conditions which promote the cultivation of acid-resistant cell rods, in particular of mycobacteria. From the positive cultures, the microbial DNA is obtained for example by means of mechanical disruption. The microbial DNA is extracted in the known way, for example by means of a QIAamp<sup>TM</sup> DNA Mini Kit (Qiagen, Hilden, Germany) from the culture isolates and, if necessary, aliquoted.

#### Example 2: PCR amplification

For amplification in optimised LightCycler<sup>TM</sup> PCR, a batch containing the ready-for-use obtainable mixture of "LightCycler FastStart DNA Master Hybridisation Probes" (Catalogue No. 239272, Roche Molecular Biochemicals) is chosen.

The following reaction mixture is produced for the LightCycler reaction:

- FastStart<sup>TM</sup> Taq polymerase
- reaction buffer
- desoxynucleoside triphosphate mixture (dNTP)
- 3 mmol/l MgCl<sub>2</sub> (end concentration)
- primer, per primer: 19 pmol corresponding to 1.1 μmol/l end concentration
- oligonucleotide FRET probe pair  
per probe: 2 pmol corresponding to 100 nmol/l end concentration

This reaction mixture is transferred by pulse centrifuging into the glass capillary of the LightCycler system and the amplification is carried out according to the "Hot Start" principle after initial denaturing at 95°C for 10 minutes with the following steps.

1. Denaturing at 95°C for 3 seconds
2. Primer hybridisation at temperatures of 68°C to 62°C for 2 seconds ("touch down annealing")
3. Polymerisation at 72°C for 40 seconds

Steps 1 to 3 are carried out 50 times in total, the hybridisation for the first 5 cycles taking place in step 2 at 68°C and during the subsequent 6 cycles the temperature being reduced to 62°C in steps of 1°C per cycle and for the remaining cycles at 62°C. The rate of temperature change is 20°C per second in all steps.

For the amplification of the region of the *narGHJI* promoter containing the DNA polymorphism specific for *M. tuberculosis*, the primer with the nucleotide sequence SEQ ID NO: 2 is used as forward primer and the primer with the nucleotide sequence SEQ ID NO: 3 as reverse primer. 155 bp fragments of the *narGHJI* promoter are amplified. It is further found that the use of these primers leads to an amplification product only in the case of pathogen strains of the *M. tuberculosis* complex but not in the case of non-tuberculous pathogens or non-mycobacterial pathogens. For this reason, the use of the primers with the nucleotide sequence SEQ ID NO: 2 and/or SEQ ID NO: 3 also allows the clear-cut distinction to be made between pathogens of the type of the *M. tuberculosis* complex vis-à-vis non-tuberculous types and vis-à-vis pathogens of non-mycobacteria.

#### Example 3: Detection and melting curve analysis

To detect the amplified fragments, FRET-labelled hybridisation probe pairs used in the reaction mixture (compare example 2) are used, one hybridisation probe partner (SEQ ID NO: 4) being associated as donor component at the 3' terminal nucleotide with fluorescein and the hybridisation probe partner which is different in each case (SEQ ID NO: 5 or 6) is associated as acceptor component on the 5' terminal nucleotide with LightCycler Red™ 640. The melting curve analysis which takes place during the detection with the hybridisation

probes immediately after the last amplification cycle begins with the denaturisation of the amplified fragments at 95°C for 30 seconds, followed by a hybridisation with the above-mentioned FRET pairs at 38°C for 30 seconds. To determine the melting curves of hybridisation, the temperature is subsequently increased continuously from 38°C to 80°C at a rate of 0.2°C per second, the fluorescence emitted by the conjugated FRET pairs being recorded continuously. The fluorescence dies out regularly as soon as at least one hybridisation probe partner melts. To evaluate the fluorescence signal, the LightCycler "run profile" program in version 3.5.3 is used, the amplification of the F2 and F3 channel of the photometric detector of the LightCycler system being automatically adjusted.

For the specific hybridisation of the region of *narGHJI* promoter containing the DNA polymorphism specific for *M. tuberculosis*, the FRET-labelled hybridisation probe pair with the nucleotide sequences SEQ ID NO: 4/SEQ ID NO: 5 is used. As an alternative, the FRET-labelled hybridisation probe pair with the nucleotide sequences SEQ ID NO: 4/SEQ ID NO: 6 is used for the specific hybridisation of the region of the *narGHJI* promoter containing the DNA polymorphism specific for *M. tuberculosis*.

Figures 2 and 3 and Table 1 show the results of the melting curve analysis.

Example 4: Comparison of the method according to the invention with the conventional nitrate reductase test

In comparative tests, different samples containing *M. tuberculosis*, *M. bovis* and *M. bovis* BCG were subjected to the method according to the invention and to the conventional nitrate reductase test (Figure 4).

The following samples were used, the sample numbers corresponding to the numbering in Figure 4:

*M. tuberculosis*:

1. H37Rv Wild type;
2. H37Rv *narG* (T-215C)
3. Erdmann Wild type;
4. Erdmann *narG* (T-15C)

*M. bovis:*

## 5. Wild type

*M. bovis BCG*

## 6. Wild type

Samples no. 1 and 3 tested positively using the method according to the invention (compare Examples 3 and 4), i.e. *M. tuberculosis* was specifically detected, a clear-cut distinction being possible between the mycobacteria strains no. 5 and 6. Samples 2 and 4, in the case of which the sequence encoding T in position -215 was modified by gene technology in such a way that it encodes C, exhibited lower values in the melting curve and comparable values in the case of samples 5 and 6 containing *M. bovis* and *M. bovis* BCG.

The biochemical test, the nitrate reductase test, confirmed the result, the non-modified *M. tuberculosis* samples no. 1 and 3 testing positively whereas no colour reaction was observed in the case of samples containing *M. bovis* and *M. bovis* BCG and the samples containing *M. tuberculosis* strains modified by T->C exchange by gene technology in position -215, which confirms the significance of the polymorphism in position -215 for the detection method according to the invention.

**Table 1**

Type	Strain	Melting temperature [°C] of the hybridisation probes specific for <i>M. tuberculosis</i>		
		Probe SEQ ID NO: 6	Probe SEQ ID NO: 5	Target region: <i>narGHII</i> pro.
		Target region: <i>narGHII</i> pro.	Target region: <i>narGHII</i> pro.	
<i>M. tuberculosis</i>	H37v ATCC 25618	62.3°	56.8°	
	Erdmann ATCC 35801	62.2°	57.0°	
	Clinical strains (n = 33)	62.2° (SD = 0.29)	56.9° (SD = 0.36)	
<i>M. africanum</i>	Clinical strains (n = 3)	57.9° (SD = 0.14)	63.4° (SD = 0.21)	
	Clinical strains (n = 2)	57.0° (SD = 0.14)	63.20° (SD = 0.28)	
<i>M. microti</i>	ATCC 19210	58.2°	63.1°	
	Clinical strains (n = 12)	58.0° (SD = 0.22)	63.2° (SD = 0.30)	
<i>M. bovis</i> BCG	Pasteur ATCC 35734	57.9°	63.6°	
	Copenhagen ATCC 27290	58.0°	63.5°	
	Moreau ATCC 35736	58.2°	63.7°	
	Tice ATCC 35743	58.2°	63.5°	
	Connaught ATCC 35745	58.2°	63.7°	
	Clinical strains (n = 4)	57.9° (SD = 0.14)	63.2° (SD = 0.26)	